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Differential Responses of Pulmonary Cells to Oxygen. DRUMMOND H. BOWDEN and IAN Y. R. ADAMSON, *Department of Pathology, University of Manitoba, Winnipeg, Canada.*

Oxygen in high concentrations is a toxic gas, and, as may be expected, the lung is the predominant target. The direct action of oxygen and the resultant pulmonary signs and symptoms are related to structural damage in the lung. Pulmonary cells are not equally susceptible to hyperoxia; a distinctive pattern of injury has been established, a differential susceptibility resembling that observed after irradiation.

Continuous inhalation of 90% oxygen by mice results, within a few days, in interstitial edema which appears to be related directly to injury to pulmonary vascular endothelium. With continued exposure for 5 to 7 days, 80-90% of the animals die; the lungs of these animals are solid and airless and the alveolar ducts and air sacs are lined with fibrin-containing hyaline membranes. This catastrophic event is a consequence of disintegration of the type 1 epithelium that lines the air sacs. Type 2 cells, alveolar macrophages, and bronchiolar cells are much less sensitive to hyperoxia, and necrosis of these cells is unusual. Throughout the exposure period the output of alveolar macrophages is unchanged; the inflammatory response to oxygen exposure is granulocytic in nature.

It has been suggested that defective synthesis of surfactant may be an important factor in the genesis of the edematous stiff lung of oxygen poisoning. The alternative explanation that lack of surfactant may be a result rather than a cause of the condition is supported by cytochemical studies suggesting that surface phospholipids are inactivated by exuded fibrinogen.

Cytodynamic studies using ^3H thymidine autoradiographs show depression of DNA synthesis during the period of exposure to oxygen, a trend that is sharply reversed within 2 days of the return to air. Between the second and third days of recovery, over 7% of alveolar cells are in the S phase of the mitotic cycle and almost 2% are in the M phase. This increase in mitotic activity is accounted for entirely by reparative proliferation of endothelial cells and type 2 epithelial cells. Within 7-10 days after removal from the oxygen chamber, the lung appears normal and the cell turnover data indicate that the proliferating type 2 cells that initially cover the injured epithelium become transformed into type 1 cells. In this model fibrosis is not seen, the animals either die or they make a complete recovery; it is an all or none situation.

The precise mechanisms of oxygen-induced pulmonary injury are unknown. There are two possible mechanisms for the injury to the endothelium, direct membrane damage by molecular oxygen diffusing across the air-blood barrier and indirect injury through elevated pO_2 levels in the blood. The sites of endothelial damage have been delineated by studying the incorporation of ^3H thymidine by endothelial cells in the recovery period. Only small, thin-walled vessels are involved; large pulmonary vessels and the endothelium of systemic vessels are not affected. This suggests that direct access of diffusing molecular oxygen is the important factor in determining the overall pattern of vascular damage in oxygen poisoning.

The question of the differential cellular reaction to oxygen remains: endothelium and type 1 cells are susceptible, whereas type 2 cells, bronchiolar cells, and macrophages are more resistant to injury. It is known that sialomucin-containing glycoproteins are an integral part of all plasma membranes and the quantity or thickness of the surface component varies a great deal in different cell types. In the lung the cells which are most susceptible to oxygen, capillary endothelium and type 1 epithelium have the thinnest layers of sialomucin.In fatal oxygen exposure, cells with thick layers, Clara cells, and type 2 cells show a reduction in this component but the cells remain intact. The finding of membrane-bound intracellular aggregates of mucopolysaccharide in type 2 cells and in surviving endothelial cells suggests a disruption of metabolism whereby sialomucin synthesized by the cell is not incorporated into the plasma membrane. Such a defect would rapidly deplete surface sialomucin and produce an abnormal plasma membrane which may be more susceptible to injury. In any event, it seems likely that the incorporation of sialomucin is critical to the integrity of cell membranes and that quantitative differences in the mucopolysaccharide component of cell membranes may be im-

portant in determining the differential susceptibility of specific pulmonary cells to oxygen-induced injury.

Effects of Long-Term, Low-Level Exposure of NO_2 or O_3 on Rat Lungs.

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The lungs of rats exposed continuously for long periods (3-5 months) to 15.0 ppm NO_2 or 0.8 ppm O_3 develop a disease that closely resembles emphysema, and the animals eventually die of respiratory failure. This animal model has been studied extensively for a number of years, (*1-5*) resulting in a relatively well-defined working hypothesis on the etiology and pathogenesis of this debilitating disease in man (*1,6*).

The early lesion is centered at the level of the terminal bronchiole and proximal alveolar duct (Figs. 1-3) and is characterized during the first 24 hr of exposure by injury and destruction of many of the ciliated cells in the terminal airways and the type 1 cells in the proximal alveolar regions, leaving the basement lamina devoid of an epithelial lining (Fig. 4).

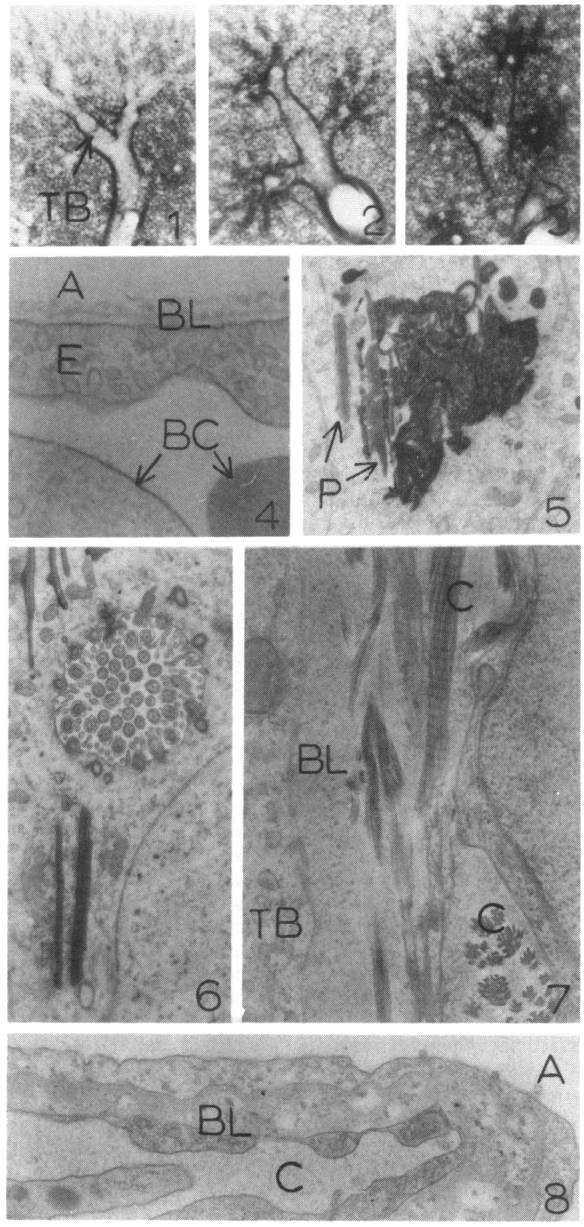
The above cell types are sensitive to injury, whereas the Clara cell in the terminal bronchiole and the type 2 cell in the alveoli are resistant (*3,5,7*). Ciliated cells that do remain lose most of their cilia and, therefore, the clearing mechanism is severely impaired (*3,7,8*).

Under continuous exposure, the destroyed and injured cells are replaced through proliferation (Figs. 2 and 3) of nonciliated cells in the terminal air-way that subsequently differentiate into ciliated cells and of type 2 cells in the proximal alveoli that become type 1 cells (*3,5,7,9,10*).

The new cells are resistant to injury, but additional cellular and tissue changes occur upon continued exposure, apparently as a result of the adapted state of the cells and their altered metabolic condition. The long-term ultrastructural changes are different for the two toxic gases, and cellular changes resulting from exposure also differ from species to species. In the rat, NO_2 produces ciliated vacuoles (Fig. 6), proteinaceous crystalloids (Figs. 5 and 6), and altered dense bodies (Fig. 5) in the cytoplasm of many of the cells in the terminal bronchioles. The basement lamina in the lesion area becomes thickened and granular (Figs. 7 and 8) and the collagen fibrils become much larger (Fig. 7). Ozone results in a greater response of fibroblasts in the lesion area (*11*), thickening of the alveolar septa, and an increase in the number of macrophages found in the proximal alveoli (*4*). These changes, together with increased mucus cause obstruction in the terminal airways, probably resulting in the structural counterpart to the "small airway disease" described by physiologists.

In the dog, chronic exposure to O_3 results in the development of squamous metaplasia in the terminal airways and a sequestering of protein in the cisternae of the rough endoplasmic reticulum of the type 2 cells in the proximal alveoli (*12*).

It appears reasonable to assume that both the obstructive material and the cellular and tissue changes described above, as well as additional factors, result in a reduced airflow and the "trapping" of air in the peripheral regions. This process contributes significantly to the etiology and pathogenesis of the disease.



FIGURES. (1) Terminal bronchiole (TB) and adjacent tissues from control animal, ×15. (2,3) Tissue density at the level of the TB and proximal alveoli is a result of proliferation for tissue repair after 48 hr exposure to 15 ppm NO_2 (2) and 0.8 ppm O_3 (3); ×15, ×15. (4) Basement lamina (BL) devoid of an epithelial layer on the alveolar surface (A) after 24 hr exposure; endothelium (E), blood cells (BC), ×13,800. (5) Dense body from the TB after long-term exposure to 15 ppm NO_2. Note the proteinaceous crystalloids (P) and swirls of membranes within the dense body; ×9,000. (6) Ciliated vacuole and proteinaceous crystalloids in cytoplasm of TB cells after long-term exposure to 15 ppm NO_2; ×9,000. (7) Thickened and granular basement lamina (BL) ad collagen (C) increased in size underlying the cells of the TB after long-term exposure to 15 ppm NO_2; ×26,900. (8) Thickening of basement lamina (BL) of capillary bed in the lesion area after long-term exposure to 15 ppm NO_2; capillary lumen (C); alveolus (A); ×14,000.

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Short-Term Effects of Ozone on Lungs of Rats, Mice, and Monkeys.

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Our studies on the short-term effects of ozone have been based mainly on exposure of 70-day-old Sprague-Dawley rats and adult rhesus and bonnet monkeys to 0.2, 0.5, or 0.8 ppm of the gas for 8 hr/day on 7 consecutive days.

Morphologic observations indicate that rats and monkeys are of approximately equal susceptibility to the short-term effects, with mild but significant lesions being consistently caused in both by levels as low as 0.2 ppm ozone. In both rats and monkeys, the predominant lesion occurs at the junction of small conducting airways and exchange regions. Specific features and location of the lesion differ in these species, however, largely because of anatomical differences. In rats, which do not have respiratory bronchioles, prominent features are accumulations of macrophages in alveolar ducts, replacement of necrotic type 1 alveolar epithelial cells from proliferating type 2 cells, and damage to ciliated and nonciliated bronchiolar (Clara) cells. In monkeys, which have well developed respiratory bronchioles, the predominant ozone-induced injury is limited to these small airways. The resulting accumulation of macrophages and shifts in alveolar epithelial cell populations are similar to those seen in rats, but there is a hyperplastic response of cuboidal, nonciliated epithelial cells lining the walls of the respiratory bronchioles. These cuboidal epithelial cells lining the walls of respiratory bronchioles of monkeys appear to have morphologic features intermediate between those of Clara cells and alveolar type 2 epithelial cells.

Yet another species variation in response is found in mice, where continuous exposure to 0.5 ppm ozone causes pronounced nodular hyperplasia of nonciliated bronchiolar (Clara) cells by the end of 7 days. This is in striking contrast to the fact that Clara cells of rats are less prominent than normal after 7 days of exposure to 0.5 ppm ozone.

Morphologic studies done in collaboration with biochemical investigations on the role of vitamin E in ozone-induced injury have shown that vitamin E deficiency potentiates the amount of damage. Vitamin E deficiency reduces the concentration of ozone at which consistent short-term effects are observed in rats. In rats fed normal diets this is 0.2 ppm, but in deficient rats equivalent effect is caused by 0.1 ppm. Deficient rats exposed to 0.8 ppm also have considerably more inflammation and fibroplasia than do rats fed the usual diet.

The phenomenon of adaptation is currently a topic of major interest because of its implications with regard to the nature and extent of lesions likely to be caused by long-term exposure to oxidant pollutants. Adaptation refers to a general process whereby cells undergo modifications in response to an altered environment. In the case of continued exposure to low levels of ozone, this is manifested by the lesion reaching a peak in 3-5 days and then, at least at 0.5 and 0.2 ppm of ozone, progressively diminishing. Our studies in rats have shown that after 90 days of 8-hr exposures there is barely detectable abnormality in a small proportion of rats at the 0.2 ppm level, minimal lesions at the 0.5 ppm level, and obvious damage at the 0.8 ppm level which is, however, less severe than after 7 days of exposure. The biochemical and morphologic changes associated with adaptation are currently being studied. One hypothesis being pursued is that insofar as the highly sensitive alveolar type 1 epithelial cells are concerned, it involves persistence of a cell type intermediate between type 1 cells and their type 2 cell precursors. The other aspects of adaptation that are of major importance are the long-term evolution of the process and the extent to which it is modified by such factors as immune status and intercurrent diseases.

Biochemical and morphological evaluations of rats exposed to 0.8 ppm ozone for 3 days and then re-exposed to ozone in the same fashion after 6, 13, or 27 days of recovery have indicated that even after 6 days of recovery the population of cells in the centriacinar regions of the lung have the same degree of susceptibility to ozone as a pre-exposure population. This indicates that continuous or repeated exposure to ozone is necessary for maintenance of adaptation.

Comparison of the Effects of Ozone and Oxygen on Lungs of Rats.

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This study was designed to compare and contrast the responses of the lung to closely related oxidant gases. Sprague-Dawley rats, CRD free, 70 ± 3 days of age, were exposed to ambient levels (0.2, 0.5, or 0.8 ppm) of ozone or 80% oxygen for periods up to one week. Levels of oxidant gas, temperature, and humidity were continuously monitored during exposure. Following exposure, rats were deeply anesthetized, killed by exsanguination, and thoracic viscera removed and fixed by airway perfusion with Karnovsky's fixative at 30 cm of H_2O pressure. Lungs were examined by light, scanning electron, and transmission electron microscopy.

Pulmonary damage caused by low concentrations of ozone occured primarily at exposed surfaces and was focused at the junction of conducting airways and exchange tissue. Changes observed within proximal alveoli included: infiltration of inflammatory cells, primarily alveolar macrophages; swelling and necrosis of occasional type 1 epithelial cells; clusters of elongated flattened epithelium, interpreted as being intermediate between type 2 and type 1 cells. Terminal bronchiole changes included: reduction in height or loss of the cytoplasmic luminal projection of Clara cells which was accompanied by an increase in short blunt microvilli projecting from the luminal surface; increased variability in length of cilia and generally shortened cilia; and clustering of basal bodies in ciliated cells suggesting ciliogenesis.

Following exposure to oxygen, the obvious light microscopic change was perivascular edema of medium and large sized vessels. Swollen endothelial cells were observed by electron mi-

croscopy. Occasional macrophage accumulations observed within alveoli did not have particular distribution within the pulmonary acinus. Clara cells appeared hypertrophic with essentially the entire cell projecting into the airway lumen. Many of these cells no longer contacted the basement membrane, and the luminal surface was uniformly smooth. Damaged ciliated cells or ciliogenesis were not features of oxygen exposure.

These morphological differences in anatomical distribution of inflammatory cells, damage to endothelium versus epithelium, and contrasting responses of Clara cells suggest different pathogenetic mechanisms of pulmonary damage by these similar oxidant gases.

Scanning Electron Microscopy of Alveolar Macrophages after Exposure to Oxygen, Nitrogen Dioxide, and Ozone.

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Alveolar macrophages (AM) obtained by tracheobronchial lavage from mice or rabbits exposed to filtered air or various levels of O_2, NO_2 and O_3 were examined by scanning electron microscopy. After exposure of mice to 60% O_2 for 2 weeks or 100% O_2 for 48 hr, the intricate surface structure of normal AM (Fig. 1*A*), consisting of fine, rufflelike membrane projections, showed distinct alterations which ranged from moderate disruption of membrane extensions to severe damage including erosion of the surface and formation of numerous blebs and fenestrae (Fig. 1*B*). This correlated with increased mortality in these exposure groups after infection with *K. pneumoniae* or influenza virus.

Examination of the effects of NO_2 on the surface structure of AM showed no changes after continuous exposure of mice for 4, 12, and 24 weeks to 0.5 ppm of NO_2 or 0.1 ppm of NO_2 with 3-hr peaks at 1 ppm for 5 days/week. Macrophages from mice continuously exposed to 2 ppm of NO_2 or 0.5 ppm of NO_2 with 1-hr peaks of 2 ppm NO_2 for 5 days/week showed distinct morphologic alterations in both treatment groups after 21 weeks that included loss of surface processes, bleb formation, appearance of fenestrae, totally denuded surface areas, and occasionally complete deterioration of the cells (Fig. 1*C*). After continuous exposure for 28 or 33 weeks, structural changes were still observed in the 0.5 to 2.0 ppm exposure group. In general, these observations could be correlated with a significant decrease in *in vitro* phagocytic activity and increase in susceptibility to infection.

When mice were exposed to 0.5 ppm of O_3, 2.5 ppm of NO_2 or the mixture of the two gases 3 hr daily 5 days/week for periods of 1 day, 1, 4, or 12 weeks, no alterations in the surface morphology or significant changes in the *in vitro* phagocytic activity of their AM were found.

Examination of AM obtained from rabbits after a single 3-hr exposure to a much higher concentration of 3 ppm O_3 showed moderate to severe structural alterations, including bleb formation and excessive fenestration (Fig. 1*D*).

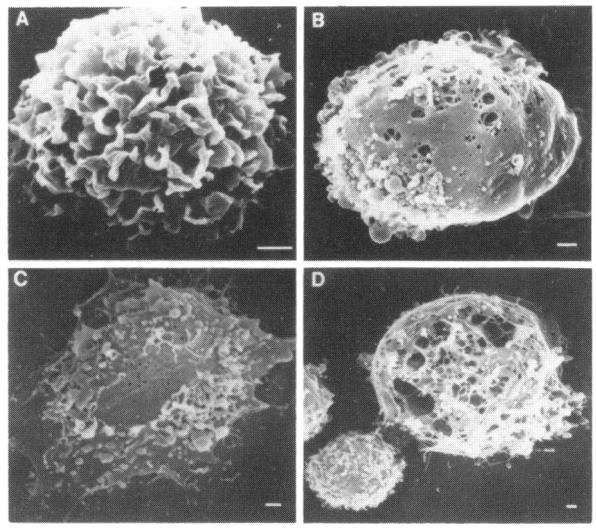

FIGURE 1. Scanning electron micrographs of alveolar macrophages obtained (*A, B, C*) from mice or (*D*) rabbit: (*A*) filtered air control; (*B*) after exposure for 48 hr to 100% O_2; (*C*) after exposure for 21 weeks continuously to 0.5 ppm of NO_2 with 1-hr peaks of 2 ppm NO_2 five times weekly; (*D*) after exposure for 3 hr to 3 ppm of O_3. The bars in the lower right-hand corner represent 1 μm.

Free Radical Reactions in Biology: Initiation of Lipid Autoxidation by Ozone and Nitrogen Dioxide.

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We have studied the initiation of the autoxidation of methyl linoleate and linolenate at 30°C by 0-2 ppm ozone and 0-5 ppm NO_2 (*1,2*). Both ozone and NO_2 act as initiators of free radical autoxidation, but by very different mechanisms. Ozone shortens the induction period (IP) but does not affect the rates of product appearance during the autoxidation phase (AP). Nitrogen dioxide increases the rates during both IP and the AP. Peroxides and conjugated diene products are formed during the IP with both ozone and NO_2 and at rates that are proportional to the concentration of the ozone or NO_2. During the IP neither linoleate or linolenate form materials which react with thiobarbituric acid (TBA) to give color at 530 nm, nor do they react with base to give color at 278 nm as does prostaglandin E (PGE). However, during the AP linolenate does form materials which give both the TBA and the PGE color tests; these materials are formed by the decomposition of a prostaglandin-like endoperoxide (*1,3*), which is produced by a radical cyclization mechanism. Conjugated diene is produced even during the IP; this indicates that autoxidation occurs during the IP, despite the presence of antioxidants, but the kinetic chain lengths are very short.

The hypothesis will be developed that most of the damage produced by these oxidants *in vivo* occurs during an IP-like phase (*4*). Therefore, the mechanisms of initiation by both ozone and NO_2 are of great interest. Initiation by NO_2 involves addition of NO_2 to the C—C double bond; our data show that the rate constants for that addition are proportional to the number of double bonds in the substrate. This addition produces a

carbon-centered free radical which reacts with oxygen to give a peroxy radical; this then abstracts the allylic hydrogen of a lipid, LH, and initiates autoxidation. Thus, NO_2 is incorporated into the substrate during the initiation process. A kinetic scheme incorporating these features predicts that the total peroxidic material (determined iodimetrically) and the conjugated dienoic material (determined by absorption at 234 nm) should both increase at rates that are proportional to the 0.5 power of the NO_2 concentration. Our present experimental data are in agreement with this scheme.

Initiation by ozone is more complex. One molecule of ozone reacts with an olefin to give (directly?) approximately one molecule of a peroxidic product (or products). The rate of formation of this product depends on the concentration of ozone and occurs despite the presence of antioxidants. It is not clear at present whether this product is produced by a radical or a nonradical mechanism; however, we are assuming as a working hypothesis that it is an ozonide (I), produced by ozone addition to the double bond of a lipid LH by the Criegee mechanism [eq. (1)].

$$O_3 + LH \longrightarrow \underset{\text{I}}{RCH\text{-}O\text{-}O\text{-}CHR} \qquad (1)$$

We have shown that ozonides are stable in our reaction system. (In the presence of transition metals, ozonides probably initiate; we have not yet tested this prediction.) Using calculated heats of reaction for various possible radical-producing reactions, we suggest that the principle initiation reaction is the molecule-assisted homolysis (MAH) *(5,6)* reaction between ozone and an unsaturated lipid molecule LH in which an allylic hydrogen is transferred [eq. (2)].

$$O_3 + LH \longrightarrow L\cdot + HO\cdot + O_2 \qquad (2)$$

If it is assumed that kinetic chain lengths during the IP are about the same for ozone and NO_2, then about 5-10% of the ozone reacts by eq.(2) to form radicals and about 90% reacts by eq.(1) to give ozonide.

This primordial radical-producing process, eq.(2), initiates short kinetic chains during the IP and leads to the production of lipid hydroperoxide, LOOH, until the antioxidant is largely used [eqs. (3) and (4)]. Then the normal chain autoxidation, initiated by LOOH and producing LOOH as a product, ensues.

$$L\cdot + O_2 \longrightarrow LOO\cdot \qquad (3)$$

$$LOO\cdot + LH \longrightarrow LOOH + L\cdot \qquad (4)$$

A kinetic treatment using these assumptions predicts that the rate of production of peroxidic materials (ozonide plus peroxides) should have an order in ozone between 0.5 and 1; our data show an apparent order of about 0.67 for 0-1.5 ppm ozone. The mechanism predicts an order in dienoic products (LOOH and LOH) of 0.5; our data give 0.41.

One striking conclusion from these studies is that autoxidation, initiated by either ozone or NO_2, occurs even in the presence of antioxidants like tocopherol or BHT. (This process produces the conjugated dienoic hydroperoxides that are observed; neither ozonolysis not NO_2 addition can produce conjugated diene.) Thus, it appears that damage could occur *in vivo* by lipid autoxidation even in systems that contain vitamin E.

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Superoxide and Superoxide Dismutase.

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The reduction of molecular oxygen occurs in biological systems via three primary routes: (1) four-electron reduction, as by cytochrome oxidase and certain other copper-containing oxidases, to produce two molecules of water; (2) two-electron reduction, as by flavoprotein and metalloprotein oxidases, to produce one molecule of hydrogen peroxide; and (3) one-electron reduction, as by certain oxidases and by the autoxidation of many low molecular weight substances, to produce the superoxide free radical, O_2^-. Thus, the metabolism of oxygen leads to the production of three products: water, hydrogen peroxide, and superoxide radical. Of these, only water is innocuous. Hydrogen peroxide is an oxidant, and superoxide can react as an oxidant, a reductant, or a free radical. Most oxygen-metabolizing organisms contain catalases or peroxidases designed to "detoxify" hydrogen peroxide, further reducing it to the level of water. Apparently all oxygen-metabolizing organisms contain superoxide dismutases to scavenge and detoxify the superoxide radical by catalyzing its dismutation *(1,2)*.

$$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2.$$

Strong correlation exists between an organism's content of superoxide dismutase and its ability to tolerate exposure to oxygen. For example, a temperature-sensitive mutant of *Escherichia coli* which lost most of its superoxide dismutase when grown at an elevated temperature concomitantly lost its ability to survive in the presence of oxygen.

In mammals, essentially all of the superoxide dismutase is intracellular, consisting of at least two distinct families of enzymes. The best characterized are the copper- and zinc-containing cytosolic enzymes. The matrix space of the mitochondria contains a soluble, manganese-containing superoxide dismutase which, in certain species, is also present in the cytosol. The relative lack of superoxide dismutase in extracellular fluids suggested that the biological components of these fluids, along with the external surfaces of cells which are exposed to these fluids, might be particularly susceptible *in vivo* to damage from the superoxide radical. Several possible *in vivo* sources of extracellular superoxide generation can be postulated. Perhaps the most significant is phagocytosing (or otherwise metabolically activated) leukocytes, shown to liberate large amounts of superoxide into the medium. Other sources may include drug-induced "oxidative stress", or in-

creased superoxide production due to increased concentration of molecular oxygen, as, for example, in a lung exposed to 100% oxygen. To assess the kinds of damage which might result when biological systems are exposed to superoxide radical, we have examined two model systems—one at the molecular level and one at the cellular level.

The object of the first study (*3*) was to examine the effects of the radical on a structural polysaccharide, hyaluronic acid, which is a common extracellular constituent. When solutions of purified hyaluronic acid were exposed to superoxide generated via xanthine oxidase plus hypoxanthine, rapid loss of viscosity occurred, indicating degradation of the macromolecule. The addition of superoxide dismutase to the system completely protected the polysaccharide from degradation. Catalase and mannitol (a good radical scavenger) were also capable of providing complete protection against depolymerization, however, indicating that superoxide *per se* was not the depolymerizing species. These data are consistent with the reaction:

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot$$

The hydoxyl radical (OH·), then, or some unidentified radical derived secondarily from it, is presumed to be the actual depolymerizing species.

The object of the second study (*4*) was to assess the cytotoxic potential of the superoxide radical. We chose to examine the polymorphonuclear leukocyte, a cell which exposes itself to a flux of superoxide when the cell is phagocytically activated. Resting (nonphagocytosing) leukocytes survive well *in vitro* for periods up to 48 hr. Phagocytosing leukocytes, however, show near normal survival for 12-15 hr, followed by a period of cell death extending over the next 20-30 hr. Inclusion of superoxide dismutase in the incubation mixture resulted in a prolonged survival of phagocytosing cells, equal to that of nonphagocytosing cells. Once again the phenomenon of protection was also observed when catalase or mannitol was included in the incubation mixture, indicating that the actual cytotoxic species was not superoxide *per se* but a radical derived secondarily from it. A peculiar feature of the phagocytically linked leukocyte death was the fact that, although superoxide production lasts only for the first 30 min of the incubation, the death did not become apparent until about 15 hr later. We found, however, that superoxide dismutase would protect the cells against subsequent death when present only during the first 1.5 hr of incubation. On the other hand, it would not protect if added after the first 1.5 hr. Thus, the free-radical damage appears to leave the cells mortally wounded, with death occurring some hours later.

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The Role of Superoxide Dismutases in Development of Pulmonry Oxygen Tolerance.

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The development of pulmonary oxygen "tolerance" in the rat is well described but poorly understood. The possible roles for the superoxide dismutases in helping produce the tolerant state have been evaluated by a series of studies.

Adult rats exposed to 85% O_2 require 5 days to develop fully the ability to survive in 100% oxygen. At the same time this occurs, the activity of pulmonary superoxide dismutase (SOD) increases about 50% (*1*). The time course for the increase in SOD closely parallels the time course for the development of tolerance. The rate at which tolerance is lost when these rats are returned to air correlates closely with the decline of SOD activity to normal levels. Guinea pigs, hamsters and mice failed to demonstrate higher activity of pulmonary SOD and also failed to acquire tolerance under similar exposure conditions (*1*).

The Cu-Zn SOD was purified from rat liver, and a rabbit antiserum specific for this SOD was prepared. An antibody titration assay was developed and used to assay the Cu-Zn SOD in homogenates from the lungs of rats made tolerant to O_2. The Cu-Zn SOD increased 42% in the tolerant animals (Crapo and McCord, Am. J. Physiol., in press). This demonstrates that the previously described increase in SOD activity is due, at least in part, to an increase in the absolute amount of the Cu-Zn enzyme rather than to an altered specific activity, or to another form of SOD.

Subcellular fractions of rat lungs have been studied for SOD activity. By the use of column chromatography and activity assays at both pH 7.8 and pH 10.0, at least two different dismutases can be demonstrated in rat lung. One of these, which differs from the Cu-Zn enzyme, is primarily located in the mitochondria. The presence of cytochrome oxidase in crude homogenates interferes with several of the common assays for SOD, but cytochrome oxidase is not active at pH 10, whereas the dismutases are. The SOD activity in mitochondrial and supernatant fractions of rat lungs was assayed by using a xanthine-xanthine oxidase assay at pH 10.0 (*2*). Results are given in Table 1.

In an attempt to modify pharmacologically the toxic effects of oxygen, SOD was administered to a group of rats every 8 hr by means of an aerosol. Significant levels of the administered SOD could be demonstrated in the lungs of the rats exposed to the aerosolized SOD. Neither method of administering SOD to rats modified the time course or the cumulative toxicity of 100% O_2 in these rats.

Table 1. SOD activity in mitochondrial and supernatant fractions of rat lungs.

	SOD activity, units at pH 10.0 [a]	
	Supernatant	Mitochondria
Controls (n=7)	2213±215	378±101
Exposed to 85% O_2 for 7 days (n=7)	3357±19 [b]	601±118 [b]

[a] All values are ± S.D.
[b] $p<0.01$.

Oxygen tolerance was compared with NO_2 tolerance in the rat. Rats require 5 days in 85% O_2 to develop full tolerance to 100% O_2, and at that point about 50% of the animals are also cross tolerant to a lethal dose of NO_2. Exposure to a sublethal dose of NO_2 (25 ppm) led to tolerance to a lethal dose of NO_2 (75 ppm for 6 hr) after only 2 days, but even after 5 days exposure, no significant cross tolerance to 100% O_2 could be demonstrated. Biochemical studies on these animals revealed that both NO_2 and O_2 led to increases in G-6-PD and catalase, but only the oxygen exposure led to significant increases in both the mitochondrial and the Cu-Zn forms of SOD (*4*).

These studies suggest that the induction of SOD in the lungs of oxygen-exposed rats is a specific adaptive mechanism which plays a major role in the development of the oxygen-tolerant state.

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Oxygen and Tissue Repair in the Lung.

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In 1972, Marino and Mitchell (*1*) reported that intraperitoneal injection of the antioxidant butylated hydroxytoluene (BHT), in mice, produced extensive cell proliferation in lung. Electron microscopic analysis of the sequence of events occurring after treatment with 400 mg/kg BHT gave the following results: 1-2 days after treatment, the cytoplasm of many type I alveolar cells becomes pale, swollen and vacuolated. In many places, type I cells desintegrate, leaving the underlying basal membrane denuded. Cellular debris are eventually engulfed by macrophages. From days 2 and 3 on, many type II alveolar cells are seen in mitosis. Their cytoplasm beigns to spread over the denuded areas. Gradually, the type II cells assume an intermediate form and, about 7 days after treatment with BHT, are transformed into type I epithelial cells. Four to five days after BHT treatment mitotic figures are also seen in capillary endothelial and interstitial cells (Hirai, et al., unpublished observations).

Tissue repair following BHT can be evaluated biochemically (*2-4*). Incorporation of thymidine into DNA and of leucine into total pulmonary protein is increased in a dose-dependent manner after BHT treatment as are the enzymes, thymidine kinase, glucose-6-phosphate dehydrogenase, and 5′-nucleotidase. Thus several biochemical indices are available to follow and to quantitate cell proliferation in lung.

The mechanism whereby BHT causes lung damage is not yet understood. Several other antioxidants tested (butylated hydroxyanisole, pyrogallol, propyl gallate, thiodipropionic acid, ascorbic acid and α-tocopherol) do not produce cell proliferation in lung. It is known that BHT may undergo, in oxygen, a nonenzymatic conversion to 2,6-di-*tert*-butyl 4-methyl 4-hydroxyperoxy 2,5-cyclohexadiene-1-one. The presence of the reactive peroxy-group makes this molecule a potentially toxic metabolite. Were this the case, oxygen might enhance BHT toxicity. Mice wer injected with BHT and placed for 8 hr in an atmosphere of 100% oxygen. This treatment had no effect upon BHT toxicity: incorporation of thymidine into pulmonary DNA, measured on days 2-6 after BHT, was not different in oxygen-exposed mice compared to animals kept in air. Thus, oxygen appears not to be involved in BHT toxicity.

Morphologic work has shown that oxygen diminishes pulmonary DNA synthesis (*5,6*). To examine whether oxygen would interfere with biochemical events accompanying tissue repair, we injected mice IP with 400 mg/kg of BHT and exposed them to 100% oxygen in a Plexiglas chamber (22 liter). Control animals were kept in an identical chamber, flushed with moistened compressed air. Since maximum DNA synthesis occurs 72 hr after BHT injection, oxygen exposures were timed so as to measure thymidine incorporation 72 hr after BHT treatment.

Exposures to 100% oxygen for 24 or 16 hr significantly diminished the *in vivo* incorporation of thymidine into total pulmonary DNA (Table 1). Shorter exposure (12, 4, or 1.5 hr) depressed DNA synthesis, but the changes were not statistically significant. Protein synthesis was reduced by 16 hr of oxygen exposure (Table 1). While there is not much DNA synthesis in the lungs of corn oil-treated animals, protein synthesis proceeds at an appreciable rate. This made it possible to measure the effect of oxygen in normal lungs: 16 hr exposures did not depress basal protein synthesis significantly. The activities of glucose-6-phosphate dehydrogenase and of 5′-nucleotidase were measured in the lungs of bothe BHT and corn oil injected animals, after exposure to air or to oxygen for 24 hr (Table 1). Oxygen did not affect basal or stimulated activities.

There is an ever-increasing number of agents including several drugs which reach the lung by routes other than inhalation and which have been found to produce lung damage, followed by cell proliferation and tissue repair (*7*). It is of considerable toxicological interest to identify and to study any possible interaction of oxygen—and possibly other oxidant gases—and tissue repair in lung, especially repair following injury produced by drugs. Areas of future investigation include: the effect of exposure to 100% oxygen on different cell types proliferating as a result of BHT treatment and the lowest concentration of oxygen which will interfere with cell repair in the lung. BHT appears to be a suitable model for these studies: lung damage may be produced by intraperitoneal injection and repair followed and quantitated with simple biochemical measurements.

Table 1. Effect of oxygen on DNA synthesis, protein synthesis, and enzyme activity in mouse lung.

Parameter	Treatment	Oxygen [a]	Air [a]
DNA synthesis,	BHT [b]	3000	6200 [c]
dpm/mg DNA	Corn oil [d]	—	—
Protein synthesis,	BHT [b]	240	330 [c]
dpm/mg protein	Corn oil [d]	120	140
Glucose-6-phosphate dehydrogenase,	BHT [e]	4.0 [f]	3.6
μmole substrate/hr/ mg of protein	Corn oil [d]	1.8	1.6
5′-Nucleotidase,	BHT [e]	1.3	1.1
μmole Pi/hr/mg of protein	Corn oil [d]	0.9	0.9

[a] Exposure for 16 hr before sacrifice for DNA and protein synthesis and for 24 hr for glucose-6-phosphate dehydrogenase and 5′-nucleotidase. $N>6$ in all cases except as otherwise noted.
[b] 400 mg/kg IP, 3 days before assay.
[c] Significantly different from 0_2-exposed animals (P=0.05).
[d] 0.1 ml/10 g IP
[e] 400 mg/kg IP, 4 days before assay.
[f] $N = 4$.

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Effects of Ozone and Nitrogen Dioxide Exposure on Lung Metabolism.

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Ozone and nitrogen dioxide are the major oxidants of photochemical smog. Most of the early studies with experimental animals have dealt with rather large concentrations of these pollutants. In recent years, however, attention is being directed toward the low-level oxidant effects.

In this study we have investigated the biochemical alterations in lung tissue after exposure of 2-month-old rats to O_3 at <1 ppm (simulating ambient conditions in photochemical smog) and to NO_2 at 5 ppm (Threshold Limit Value). The metabolic parameters studied include O_2 consumption, glucose utilization, pyruvate and lactate production, and protein and lipid biosynthesis in tissue slices; substrate utilization in homogenate; and marker enzyme activities in subcellular fractions (viz., mitochondrial succinate oxidase, succinate-cytochrome c reductase; microsomal NADH- and NADPH-cytochrome c reductases; and cytosolic glucose-6-phosphate dehydrogenase, glutathione reductase, disulfide reductase, glutathione peroxidase and gluthathione-disulfide transhydrogenase). Results shown below are from O_3 exposures, but similar trends were obtained from limited exposures conducted using NO_2.

As judged from enzyme activities, lung metabolism may exhibit an initial depression within 24 hr of exposure, and a subsequent elevation which attains a peak between 3 and 4 days of exposure. Table 1 presents results for some of the marker enzyme activities. For exposures involving 0.8 ppm O_3, a 31-85% elevation of metabolic parameters has been observed. For lower levels of O_3 (viz., 0.5 and 0.2 ppm) the changes in metabolic parameters are relatively small, exhibiting a fairly dose-dependent response. Prolonged exposures to O_3 (viz., 0.8 ppm) show that the biochemical changes in the lung remain elevated for as long as 30 days of exposure. If the exposure is discontinued after a few days, the biochemical changes in recovering animals return to control levels within a week.

It may be concluded that low-level oxidant exposures cause biochemical changes in the lung. The augmentation of biochemical parameters may be a reflection of lung tissue injury caused by oxidant exposure, and the tissue repair processes, including cell renewal and/or proliferation and synthesis of cellular components, may be contributory to this augmentation. The biochemical changes, which reach a plateau within a few days, seem to follow a similar time-course as the morphological changes, i.e., the cellular proliferation in the lung during low-level oxidant exposures also develops in approximately 3 days. Morphological and biochemical changes evoked during the first 3-4 days of oxidant exposure possibly bring about a state of adaptation whereby a continued exposure results in little or no immediate damage to lung tissue, and hence the enzyme activities reach a plateau. The return of metabolic activities in lung tissue toward control values a few days after termination of O_3 exposure may be ascribed to a cessation of injury and a relative completion of the reparative process. Biochemical changes in the lung resulting from low-level oxidant exposure are therefore reversible.

Table 1. Biochemical changes in lung after 0.8 ppm O_3 exposure.

Biochemical parameters	Increase over control, % [a] After 2 days exposure	After 4 days exposure
Oxygen consumption [b]	21 ($p>0.05$)	31 ($p<0.05$)
Succinate oxidation [c]	15 ($p>0.05$	51 ($p<0.01$)
Glycerol-1-P oxidation [c]	10 ($p>0.05$)	34 ($p<0.05$)
Glucose-6-P dehydrogenase [d]	45 ($p<0.05$)	85 ($p<0.01$)
Glutathione peroxidase [d]	26 ($p<0.05$)	70 ($p<0.01$)

[a] Expressed per lung.
[b] Tissue slices.
[c] Homogenate.
[d] Cytosol.

Experimental Studies on Human Health Effects of Ozone.

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Development of tolerance in laboratory animals exposed repeatedly to relatively high concentrations of ozone (O_3) and other oxidant gases is well known. Animals exposed to a sublethal concentration of O_3 develop the ability to survive subsequent exposures at normally lethal concentrations and do not develop pulmonary edema as readily as do previously unexposed animals. Tolerance does not appear to protect against cytotoxic effects of O_3 or against chronic pulmonary changes after prolonged xposures, however. That adaptation can develop in humans exposed repeatedly to photochemical smog has been suggested by analogy with animal tolerance, although exposure concentrations and health effects experienced by humans are much less than in animal studies. Little information exists concerning the possibility of adaptation in humans, even though this is a potentially important consideration in setting air quality standards to protect health.

Comparison of several previous studies of O_3 effects in humans suggests that Los Angeles residents, frequently exposed to photochemical smog, may experience less severe clinical and physiological reactions to O_3 exposure than residents of areas with less photochemical pollution (Hackney et al., Arch. Environ. Health, in press). This possibility was investigated further by comparing responses to controlled O_3 exposures in Los Angeles area residents ($\geq$ 3 yr in area) and new arrivals to Los Angeles (< 5 days in area before study). The investigation was conducted near the end of the summer smog season to maximize opportunity for adaptation development in residents. Six residents (all female, one smoker) and nine new arrivals (7 female nonsmokers, 1 male nonsmoker, 1 male smoker) were studied; all were preprofessional students, aged 21-25, with normal baseline pulmonary function tests. Each subject was exposed on one day to purified air (control) for 2 hr

with intermittent light exercise, and on the following day to 0.4 ppm O_3 in purified air under the same conditions. Exposure temperature was 31°C and relative humidity 35%. Pulmonary function tests were performed at the end of the exposure period, and respiratory symptoms during and following exposure were investigated using a standardized symptom questionnaire. Resident and new-arrival groups did not differ significantly in symptoms reported, although new arrivals more often reported increased symptoms after exposure relative to control. The new arrival group showed significant (<0.05) losses in forced vital capacity (FVC), 1-sec forced expiratory volume (FEV_1) and maximum mid-expiratory flow rate (MMF) after exposure; the resident group showed no significant changes in any of these. The mean changes with exposure differed significantly between groups (<0.05) for FEV_1 and MMF. Neither group showed significant decrement in closing volume or delta nitrogen after exposure.

These results are consistent with previous observations in suggesting that adaptation to acute respiratory effects of O_3 takes place in Los Angeles residents. Biochemical indicators of the adapted state have not been found thus far, nor is the nature of the adaptation mechanism or its relationship (if any) development of chronic lung disease understood.

Biochemical Changes in Human Blood Following Ozone Inhalation.

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Evidence of biochemical changes were sought in blood taken from healthy human adults immediately following an acute exposure to low levels of ozone (O_3). The experiments were performed in an environmental chamber capable of temperature and relative humidity control, and also capable of freeing incoming air of detectable gaseous and particulated pollutants. Subjects entered the environmental chamber and breathed either purified air (sham exposure) or purified air to which O_3 was added. Levels of O_3 were at or below those encountered in Los Angeles smog during air pollution alert episodes. Subjects underwent intermittent exercise sufficient to double their minute volume. Each subject served as his own control and paired group statistical analyses were performed on biochemical studies of erythrocytes and sera.

Subjects were exposed to 0.5 ppm O_3 in the first experiment ($n = 6$). Statistically significant changes ($p \leq 0.05$) occurred at the erythrocyte membrane and intracellular levels. The O_3 exposure increased the erythrocyte membrane's sensitivity to a hemolytic challenge by 2% H_2O_2, while the activity of the membrane enzyme acetylcholinesterase (AcChase) was decreased. Intracellular reduced glutathione (GSH) was decreased while the enzymes glucose-6-phosphate dehydrogenase (G6PDH) and lactate dehydrogenase (LDH) were significantly increased. Serum vitamin E and malonaldehyde (lipid peroxidation product) were both significantly increased. Some subjects had moderate to severe clinical symptoms upon exposure to 0.5 ppm O_3 so subsequent studies were performed at 0.37-0.4 ppm levels of the oxidant.

The blood biochemical responses following inhalation of 0.37-0.4 ppm O_3 ($n = 26$) were qualitatively similar to those detected following exposure to the higher oxidant level, but were generally not as great. A statistically nonsignificant increase was detected in erythrocyte membrane fragility to H_2O_2 while a statistically significant decrease occurred in AcChase activity. Other statistically significant changes included a decrease in GSH, an increase in G6PDH and LDH activities. A nonsignificant increase in serum vitamin E was detected, while no evidence of lipid peroxidation was found.

A limited number of biochemical studies were performed on blood of Los Angeles residents (possibly O_3 adapted) and nonresidents following O_3 exposure. Because they tend to show greater biochemical changes, this evidence suggests that nonresidents may be more sensitive to O_3. The number of observations was too small however to draw valid statistical inferences.

In summary, significant biochemical changes were demonstrated in blood from human volunteer subjects following inhalation of O_3 relative to O_3-free conditions.

Anticarcinogenesis Studies with Vitamin A and Its Analogs.

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Vitamin A and its synthetic analogs (retinoids) control cell differentiation in many epithelial tissues which are primary sites of occurrence of cancer in man. The mechanism of action of control of cell differentiation by retinoids resembles that of steroid hormones, such as estrogens and androgens. The effects of retinoids on normal and preneoplastic cell differentiation can be studied in organ culture. Feeding of natural retinoids at high dietary levels can partially prevent chemical carcinogenesis in epithelia of bronchi, trachea, stomach, uterus, skin, and breast of epxerimental animals. The effectiveness of natural retinoids for prevention of cancer in man, however, is limited by inadequate tissue distribution or excessive toxicity. New synthetic retinoids, which have different profiles of tissue distribution and lesser toxicity compared to natural retinoids, are thus being evaluated for greater effectiveness in prevention of cancer at several different epithelial sites.

Lipid Peroxidation in Animals and Man.

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Recent evidence indicates that the oxidative decomposition of lung unsaturated fatty acids is a major mechanism of ozone toxicity and that this occurs in animals exposed to concentrations of ozone which are found in areas experiencing significant photochemical air pollution. There is also ample evidence suggesting that vitamin E deficiency enchances this toxic effect of ozone. However, before concluding that these findings warrant recommending the ingestion of vitamin E by populations exposed to ozone, more information must be obtained. Our studies have been designed to indicate whether lipid peroxidation actually occurs as a pathological entity in the absence of vitamin E deficiency in man (Goldstein and McDonagh, J. Clin. Invest, 57: 1302, 1976).

A spectrofluorescent technique developed by Tappel and his colleagues was utilized to evaluate lipid-containing extracts of red cells freshly obtained from patients receiving the oxidant hemolytic drug diaminodiphenylsulfone (DDS). Red cell membrane lipid peroxidation was previously suggested to occur in DDS-induced hemolysis, but the evidence was based solely on *in vitro* findings. In eight patients with normal serum vitamin

E levels who received hemolytic levels of DDS we observed an increase in fluorescence consistent with the covalent crosslinking of malonaldehyde, a lipid peroxide breakdown product, to lipid amino groups. The fluorescence was particularly notable in older red cells suggesting an accumulation of such crosslinks during the lifetime of the circulating red cell. *In vitro* exposure of red cells to ozone produced similar fluorescence.

The potential consequences of the formation of malonaldehyde-amino group crosslinks was further evaluated. Following ozone-induced lipid peroxidation or incubation with malonaldehyde *in vitro*, an increase in apparent red cell membrane protein viscosity was observed utilizing a technique in which the ability of chloroform to quench native protein fluorescence provides an index of cell membrane fluidity. Such increased viscosity would presumably interfere with cell membrane function. In addition, incubation of histidine-requiring auxotrophs of *S. typhimurium* with malonaldehyde resulted in an increase in reversion rate in certain strains. (Mukai and Goldstein, Science 191:868, 1976). The findings are consistent with a crosslinking of DNA by malonaldehyde, suggest that lipid peroxidation may be mutagenic, and provide a potential mechanism by which ozone inhalation may produce lymphocyte chromosome abnormalities.

Several other questions remain to be investigated. These include: (1) the extent, if any, to which supranormal vitamin E levels are more efficacious than normal vitamin E levels in protecting against ozone-induced lung lipid peroxidation; (2) whether cellular membranes are in fact the primary sites of ozone toxicity; (3) the nature of the relationship of lung vitamin E levels to ingested vitamin E, to blood levels, and to adipose tissue stores; (4) the existence of long-term toxic effects due to chronic vitamin E ingestion; and (5) the nature of the relationship of vitamin E to other antioxidant defense mechanisms possibly protective against ozone toxicity.

Vitamin E and Selenium-Glutathione Peroxidase in Protection Against Oxidant Damage.

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Among types of biochemical damage to the lung by environmental oxidants, lipid peroxidation is a major mechanism. Measurements of lipid peroxidation provide quatitative chemical information about lung damage. Fluorescent product measurements are not applicable to short-time studies of damage. Measurement of thiobarbituric acid-reacting products have been successfully applied in monitoring vitamin E protection (*1*). Increases in glutathione peroxidase activity appear to correlate with lipid peroxidation in lung. Increases in glutathione peroxidase in rat lung as a function of exposure to O_3 in the range of 0.2 to 0.8 ppm indicate that lung damage is proportional to the O_3 concentration and that there is no lower threshold of O_3 that is not damaging (*2*). These biochemical methods for the measurement of increased glutathione peroxidase and thiobarbituric acid-reacting products require use of large numbers of experimental animals because of the variability within animals. Experiences with smaller numbers of primates indicated the need for measurements that are nondestructive and that can be repeated on the same animals as a function of experimental variables. Ethane and pentane are among the hydrocarbon gases produced in small amounts from lipid peroxidation and peroxide decomposition. Iron-catalyzed decomposition of hydroperoxides yields approximately 0.002 mole ethane/mole of linolenate hydroperoxide and 0.005 mole pentane/mole of linoleate hydroperoxide. Methods for measuring hydrocarbon gases from experimental animals and humans are being developed. Gas chromatography on alumina columns allows measurement of 0.5 ppm ethane and pentane. Vitamin E-deficient rats exposed to 6.5 ppm O_3 during 1 hr showed significant increases in ethane and pentane output.

The two natural protectors against peroxidative damage are vitamin E and selenium-glutathione peroxidase. Vitamin E at nutritional and prophylactic levels is protective against acute O_3 and NO_2 toxicity and against low level O_3 damage (*3*). In contrast, vitamin E-deficient rats are much more labile and are more damaged by exposures to O_3 and NO_2. Increased intakes of vitamin E should be considered for human protection against exposure to oxidants in the air. The newly discovered selenium-glutathione peroxidase system is an important protector system for lung. Animal lungs and RBC have relatively high activity of glutathione peroxidase, which provides protection against any lipid hydroperoxides formed. Glutathione peroxidase is produced in proportion to selenium in the diet. Selenium appears to be covalently bound in a hydrophobic active site (*4*). At low dietary selenium intake, rats exposed to 0.8 ppm O_3 showed evidence of more damage than rats with higher dietary selenium.

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Antioxidants and Lung Disease.

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The oxidizing air pollutants, NO_2 and O_3, catalyze the autoxidation of unsaturated fatty acids. The autoxidation can be inhibited by phenolic antioxidants. Vitamin E is as effective as other phenolic antioxidants. Mice and rats deprived of dietary vitamin E are more susceptible to the toxic effects of both air pollutants than those animals given large amounts of vitamin E (100 mg/kg of diet). Although mortality was delayed, rats receiving vitamin E supplements still died. Fatty acid ozonides are potential intermediaries in the oxidation of unsaturated fatty acids by O_3. Ozonides catalyze the autoxidation of tissue and red cell lipids. Hemoglobin is also oxidized in red cells leading to Heinz bodies. Heinz bodies are probably the result of disulfide linked polymers of hemoglobin promoted by fatty acid ozonides. In man, supplementation with 100-200 mg of *dl-α*-tocopheryl acetate prevented Heinz body formation in a dose-response related manner. Ozonides and O_3 promote inflammatory responses. Polymorphonuclear leucocytes are mobilized and such a mobilization may be due to a chemotactic property of partially oxidized fatty acids. O_3 exposure inhibits lung cyclooxgenase activity and PGE_2 15-ketodehydrogenase but fails to alter the kinetics of PGE_2 uptake and release by the lung. These data support the concept that oxidation of unsaturated fatty acids occurs on O_3 and NO_2 inhalation. The products of oxidation are biologically potent. The natural systems for the oxidation of unsaturated fatty acids to prostaglandins and thromboxanes are also inhibited, possibly by nonspecific fatty acid peroxides. The deleterious effects of such events can be inhibited in part by appropriate biological antioxidants. Vitamin E is most likely the most promising prophylactic agent for man.